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(54) Title: DENDRIMERIC POLYCHELANTS

(57) Abstract

There are provided polychelants and their metal chelates which are useful in diagnostic imaging and in radiotherapy and which comprise a plurality of macrocyclic chelant moieties, e.g. DOTA residues, conjugated to an up to fifth generation dendrimer backbone molecule, e.g. a starburst dendrimer. To produce a site-specific polychelate, one or more of the macrocyclic chelant carrying backbone molecules may be conjugated to a site-directed molecule, e.g. a protein.

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DENDRIMERIC POLYCHELANTS

This invention relates to low generation dendrimeric polychelants, as well as the corresponding bifunctional polychelants, site-directed macromolecular conjugates of macrocyclic chelants, and the chelates and salts thereof and their applications in medicine, including the field of diagnostic imaging.

The polychelates are especially suited for use in enhancing images of selected mammalian organs, tissues, cells, and the like, in vivo, using Magnetic Resonance Imaging (MRI), X-ray, gamma scintigraphy, and CT scanning, by virtue of their enhanced imaging properties and site specificity. The polychelants are also particularly well suited for use as intravascular contrast agents, blood pool agents, in these imaging modalities. As such they may be used in imaging blood vessels, e.g. in magnetic resonance angiography, in the measurement of blood flow and volume, in the identification and characterization of lesions by virtue of differences in vascularity from normal tissue, in the imaging of the lungs for the evaluation of pulmonary disease and in blood perfusion studies. The polychelants are also well suited for metal detoxification, therapeutic delivery of radioisotopes and diagnostic nuclear medicine applications.

Medical imaging modalities, such as MRI, X-ray, gamma scintigraphy, and CT scanning, have become extremely important tools in the diagnosis and treatment of illnesses. Some imaging of internal parts relies on inherent attributes of those parts, such as bones, to be differentiated from surrounding tissue in a particular type of imaging, such as X-ray. Other organs and

anatomical components are only visible when they are specifically highlighted by particular imaging techniques.

One such technique with potential to provide images of a wide variety of anatomical components involves biotargeting image-enhancing metals. Such a procedure has the possibility of creating or enhancing images of specific organs and/or tumors or other such localized sites within the body, while reducing the background and potential interference created by simultaneous highlighting of non-desired sites.

Researchers have recognized for many years that chelating various metals increases the physiologically tolerable dosage of such metals and so permits their use in vivo to enhance images of body parts (see for example C.D. Russell and A.G. Speiser, J. Nucl. Med. 21: 1086 (1988) and U.S. Patent No. 4,647,447 (Gries et al.)). However, such simple metal chelate image enhancers, without further modification, do not generally provide any particularly significant site specificity.

The attachment of metal chelates to tissue or organ targeting molecules, e.g. biomolecules such as proteins, in order to produce site specific therapeutic or diagnostic agents has been widely suggested.

Many such bifunctional chelating agents, i.e. agents which by virtue of the chelant moiety are capable of strongly binding a therapeutically or diagnostically useful metal ion and by virtue of the site-specific molecular component are capable of selective delivery of the chelated metal ion to the body site of interest, are known or have been proposed in the literature. Thus for example even relatively early publications in the field of MRI contrast agents, such as GB-A-2169598 (Schering)

and EP-A-136812 (Technicare) suggested the use as contrast agents of paramagnetic metal ion chelates of bifunctional chelants.

The attachment of chelant moieties to site-specific macromolecules has been achieved in a number of ways, for example the mixed anhydride procedure of Krejcarek et al. (Biochemical and Biophysical Research Communications 77: 581 (1977)), the cyclic anhydride procedure of Hnatowich et al. (see Science 220: 613 (1983) and elsewhere), the backbone derivatisation procedure of Meares et al. (see Anal. Biochem. 142: 68 (1984) and elsewhere - this is a technique used by Schering in EP-A-331616 to produce site specific polychelates for use as MRI or X-ray contrast agents), and the linker molecule procedure used for example by Amersham (see WO-A-85/05554) and Nycomed (see EP-A-186947 and elsewhere) to produce paramagnetic metal ion chelates of bifunctional chelants for use as MRI contrast agents.

Thus, Krejcarek et al (supra) disclosed how polyaminopolycarboxylic acid (PAPCA) chelants, specifically DTPA (diethylenetriaminepentaacetic acid) could be conjugated to a protein, such as human serum albumin (HSA), by reaction of the triethylamine salt of the PAPCA with isobutylchloroformate (IBCF) and by reacting the IBCF-PAPCA adduct with the protein. Their aim was to attach one radioactive metal per human serum albumin molecule for the purpose of measuring biological function.

Site specific uses of various imaging techniques all require or would be enhanced by use of a multiplicity of the appropriate metal ion conjugated to a site-directed macromolecule. For example, it is believed that a 50% reduction in T_1 relaxation time of

water protons in a target tissue is a requirement for an effective MRI contrast agent. Considering the affinity of antibodies for their antigens and the concentration of these antigens in the target tissues, it has been calculated that each antibody molecule must carry a number of paramagnetic centers to bring about these levels of T_1 reduction. (see Eckelman, et al., NATO ASI Series, Series A, 152:571 (1988)).

Unger et al. in Investigative Radiology 20:693 (1985) analyzed tumor enhancement for magnetic resonance imaging using an anti-CEA monoclonal antibody conjugated with Gd-DTPA. They found no tumor enhancement when 4 Gd atoms were bound per antibody molecule, and predicted that a far greater ratio of imaging metal atoms per macromolecule would be required to be effective.

Likewise, Schreve and Aisen in Mag. Res. in Medicine 3:336 (1986), concluded that the concentrations of paramagnetic ion which could be delivered to a tumor using the described technology would result in large doses for humans, making this approach to imaging highly limited in its use.

For site specific image enhancement however it is important that the site specificity of the tissue or organ targeting moiety of such chelates of bifunctional chelants should not be destroyed by conjugation of the chelant moiety. Where the bifunctional chelant contains only one chelant moiety this is not generally a severe problem; however when attempts have been made to produce bifunctional polychelants by conjugating several chelant moieties onto a single site-specific macromolecule, it has been found not only may the maximum achievable chelant: site-specific macromolecule ratio be relatively limited but as the ratio achieved increases the site-specificity of the resulting bifunctional polychelant

decreases.

Numerous attempts have nonetheless been made to produce bifunctional polychelants with increased numbers of chelant moieties per site-specific macromolecule.

Thus Hnatowich et al. (supra) used the cyclic anhydride of the chelant DTPA to attach it to a protein.

This is a relatively simple one-step synthesis procedure which as a result has been used by many other researchers. However, due to the presence of two cyclic anhydride groups in the starting material, widespread cross-linking of the macromolecules can lead to the production of conjugates that can not readily be characterized (see Hnatowich et al., J. Immunol. Methods 65:147 (1983)). In addition, this procedure suffers from the same drawback as that for Krejcarek's mixed anhydride method in that the uncontrolled addition of more than a few chelant moieties destroys the site-specificity of the macromolecule to which they are linked. (See also Paik et al. J. Nucl. Med. 25:1158 (1983)).

In order to overcome the problems of attaching larger numbers of chelant moieties to a site-specific macromolecule without destroying its site-specificity, i.e. without disturbing its binding site(s), there have been many proposals for the use of a backbone molecule to which large numbers of chelant moieties can be attached to produce a polychelant one or more of which can then be conjugated to the site-specific macromolecule to produce the bifunctional polychelant.

The by now conventional cyclic anhydride conjugation technique of Hnatowich et al. (supra) has thus been used to produce bifunctional polychelants in

which the chelant moieties are residues of open chain PAPCAs, such as EDTA and DTPA, and in which the backbone molecule is a polyamine such as polylysine or polyethyleneimine. Thus for example Manabe et al. in Biochemica et Biophysica Acta 883: 460-467 (1986) reported attaching up to 105 DTPA residues onto a poly-L-lysine backbone using the cyclic anhydride method and also attaching polylysine-polyDTPA polychelants onto monoclonal antibody (anti-HLA IgG_1) using a 2-pyridyl disulphide linker achieving a substitution of up to about 42.5 chelants (DTPA residues) per site-specific macromolecule. Torchlin et al. in Hybridoma 6:229-240 (1987) also reported attaching DTPA and EDTA to polyethyleneimine and polylysine backbones which were then attached to a myosin-specific monoclonal antibody, or its Fab fragment, to produce bifunctional polychelants for use in MRI or scintigraphy.

While Manabe and Torchlin have reported the production of bifunctional polychelants, the cyclic anhydride route adopted by Manabe poses cross-linking and hence characterization problems and Torchlin et al in their conclusion doubted that their technique would enable the paramagnetic metal concentration to be increased sufficiently to permit MRI of tumours.

Sieving et al. in WO-A-90/12050 disclosed techniques for producing polychelants comprising macrocyclic chelating moieties, such as polylysine-polyDOTA, and for the preparation of corresponding bifunctional polychelants. Sieving et al. also suggested the use of starburst dendrimers, such as the sixth generation PAMAM starburst dendrimer of Tomalia et al. (see US-A-4587329 and Polymer Journal 17:117 (1985)), as the skeleton for such polychelants.

The present invention lies in the recognition that

low generation dendrimers carrying macrocyclic chelating moieties are a particularly suitable form of polychelant for diagnostic and therapeutic use by virtue both of their structures and of their substantial uniformity in terms of molecular weight distribution and metal loading capacity. By virtue of their non-particulate nature and relatively high molecular weights these low generation dendrimeric polychelants can function as blood pool agents without requiring attachment to site-directed biomolecules.

Thus viewed from one aspect the invention provides a polychelant comprising an up to fifth, especially an up to fourth, generation dendrimeric backbone moiety with linked thereto a plurality of macrocyclic chelant moieties capable of complexing metal ions, and metal chelates and salts thereof.

A dendrimeric (or cascade) polymer, such as that forming the backbone moiety in the polychelants of the invention, is formed using monomers which act as branching sites and with each successive branching a new "generation" oligomer is formed.

The dendrimeric polychelants of the invention and the chelates and salts thereof are here termed "magnifiers". The chelant moieties in the magnifiers are capable of chelating metal ions with a high level of stability, and are metallated with the appropriate metal ion(s), eg to enhance images and/or to deliver cytotoxic doses of radioactivity.

If desired, the magnifiers can be attached by well-known methods to a site-directed molecule, e.g. a protein, to form bifunctional polychelants which can enhance images and/or deliver cytotoxic doses of radioactivity to the targeted cells, tissues, organs,

and/or body ducts. Alternatively the magnifiers may be used as blood pool agents without being coupled to site directed molecules.

The magnifiers are in and of themselves useful entities in medical diagnosis and therapy, due in part to their unique localization in the body. The monomeric chelates presently used for MRI contrast enhancement (e.g., $Gd(DTPA)^{2-}$, $Gd(DOTA)^{1-}$) have <u>in vivo</u> applications related to their specific, rapid biodistribution, localizing these chelates in the extravascular/ extracellular spaces of the body. The size of the magnifier, typically 1 to 100 kD, especially 5 to 90 kD, more especially 20 to 90 kD, particularly 30 to 85 kD, eg 30 to 50 kD, radically alters the biodistribution of the chelates. The magnifiers generally have extended intravascular residence times, generally of the order of hours, and usually will eventually clear into the extracellular fluid (ECF) space and undergo renal excretion. Thus as these magnifiers remain primarily in the intravascular system for a diagnostically useful residence time, they are suitable for a range of uses from blood pool and cardiac perfusion imaging, CNS tumour detection and volume determination to thrombus detection and angiography. As blood pool agents they are particularly suited to use in studies of blood flow or volume, especially in relation to lesion detection and myocardial perfusion studies. The conventional monomeric MRI contrast agents which rapidly disperse into the extracellular/extravascular space cannot readily be used for these purposes. Moreover in view of their enhanced relaxivity, the MRI contrast agents according to the invention can be administered at significantly reduced dosages relative to current monomeric MRI contrast agents such as GdDTPA and GdDOTA, thus providing a significantly improved safety margin in their use.

The invention also enables water-soluble MRI contrast agents to be produced which can safely be administered orally for efficient liver imaging. For such agents the dendrimeric polychelant would preferably be used as the vehicle for Mn(II) or Gd(III) paramagnetic ions for optimum MR efficiency.

Furthermore, by suitable selection of chelated species, chelates according to the invention may be produced which are capable of functioning as X-ray agents (for example by choosing tungsten) and also as both MR and X-ray contrast agents by choosing an appropriate lanthanide metal ion.

Attachment of the magnifier to a site-directed molecule results in even greater in vivo target specificity. The molecule is preferably an antibody, antibody fragment, other protein or other macromolecule which will travel in vivo to that site to deliver the chelated metals. In the present invention the capacity of this site-directed macromolecule to travel and/or bind to its target is not compromised by the addition of the chelated metals. The number of chelates per molecule is sufficient to enhance the image of that particular target. The resulting bifunctional polychelates are distinct entities, and desirably are substantially non-crosslinked.

In one embodiment the magnifiers of the invention can be represented by the formula I

$$B(L)_n \qquad (I)$$

where B is the residue of a dendrimeric backbone molecule, eg a polyamine, typically a molecule containing terminal amine groups extending radially outwards from a central core moiety;

each L is independently the residue of a macrocyclic chelant (or a chelate or salt thereof);
and n is an integer in the range of 3 to 200, preferably up to 100, eg up to 50.

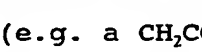
Using this formula for the magnifiers, the corresponding bifunctional polychelants and polychelates of the invention can be represented by the formula II

$$T\ (B'(L)_n)_m \qquad (II)$$

where T is the residue of a site-directed molecule, each $B'(L)_n$ is independently the residue of a magnifier of formula I, optionally incorporating a residue X' of a linker molecule which serves to link the magnifier to the molecule, and m is a positive integer, e.g. 1 to 10, preferably 1,2,3,4 or 5.

The dendrimeric backbone molecule to which the macrocyclic chelants are bound preferably has a multiplicity of amines arranged to extend radially outwards from a central core moiety, i.e. a starburst dendrimer-type backbone molecule. Such starburst dendrimer-type backbone molecules comprise a central core moiety to which a plurality of linker groups are attached. These linker groups may either be bonded directly to the macrocyclic chelates or may, optionally and preferably, be terminally branched by the addition of further linking moieties which may each be the same or different to the first linker groups. A backbone molecule wherein a central branched linker group has itself been terminally branched once is termed a first-generation ($G_{1.0}$) backbone molecule. Further terminal branching of the linker groups of first-generation backbone molecules will provide second, third, fourth, fifth, etc. generation backbones. With each successive round of branching, the number of attachment points

available for bonding the macrocyclic chelant groups increases.

The linkage between the backbone B and the macrocyclic chelant moiety is preferably via an amide bond, the amide nitrogen deriving from the backbone molecule and the amide carbonyl group deriving from a carboxyl or carboxyl derivative functionality on the macrocyclic chelant. Particularly preferably the macrocyclic chelant is a PAPCA and especially preferably the carboxyl or carboxyl derivative functionality is attached to the or a ring structure of the macrocyclic chelant at a donor ring heteroatom, especially a nitrogen. As an alternative however, the dendrimeric backbone may be linked to the macrocyclic chelant moiety via a linker group attached to the macrocyclic chelant at a ring heteroatom (e.g. a CH_2CONH-alk-  -X"' group where alk is a C_{1-4} alkylene chain and X"' is NCS, N_2^+, NCO, -alk-COOH, $NHCOCH_2Cl$ or $NHCOCH_2Br$) or at a ring carbon, e.g. as suggested by Meares et al. (see Acc Chem Res 17:202 (1984) and US-A-4678667).

Magnifiers and bifunctional polychelants can be used in their unmetallated or undermetallated state for absorption of available metal ions in vivo, such as in metal detoxification. Alternatively, magnifiers and bifunctional polychelants can be used in their metallated form to deliver chelated metal ions for diagnostic or therapeutic applications.

Metal ions are chosen for chelation by the magnifiers for their ability to perform their diagnostic or therapeutic role. These roles include but are not limited to enhancing images in MRI, gamma scintigraphic or CT scanning, or X-ray, or delivering cytotoxic agents to kill undesirable cells such as in tumors.

For use with radionuclides, such as in nuclear medicine, this invention provides the advantage of tight binding of the radionuclides by the macrocyclic chelants. This allows a more specific image due to lower background levels of the metals.

Preferably, metal incorporation into bifunctional polychelants is accomplished prior to attachment of the magnifier(s) to a site-directed molecule. The metal is titrated from sub-stoichiometric levels up to full incorporation, thus eliminating the need for dialysis and extensive chromatographic purification. In this manner significant losses as well as dilution are avoided. Non-specific binding of the metal ions to the site-directed molecules is also prevented. However, application of the invention to radionuclides with short half-lives may require metallation of the bifunctional polychelant as a final step, followed by simple rapid purification (e.g. gel filtration) to remove excess unbound radionuclide.

In the bifunctional polychelant, preferably one or two backbone molecules are linked to the site-directed molecule. By limiting the number of magnifiers linked to the site-directed molecule, the pharmacological behavior of the bifunctional polychelant would be expected to show high target specificity and low non-specific binding.

The bifunctional polychelants are capable of containing a large number of macrocyclic chelant moieties. This allows site-specific imaging to be enhanced beyond the levels previously available.

These magnifiers and bifunctional polychelants are not only extremely useful for magnetic resonance imaging, they are also useful in other forms of imaging,

as well as in nuclear medicine. Osmolality of currently available image enhancing agents contributes to some of the undesirable side effects of these agents, including pain to the patient. By allowing a marked increase in the number of image enhancing chelated metal centres per molecule in solution, this invention allows for a significant decrease in osmolality, while retaining the same level or increasing the level of image enhancement.

The magnifiers of the invention are produced by conjugating a plurality of macrocyclic chelants onto a dendrimeric backbone molecule, generally a water-soluble polymer having reactive groups. The backbone polymer will conveniently have at least 3 and preferably up to 96, eg up to 48, reactive groups. The backbone molecule conveniently is a starburst dendrimer. The reactive groups can be amines, preferably primary amines, carboxylates, alcohols or thiolates etc.

The starburst dendrimers include polyaminoamido dendrimers (PAMAM) and related starburst dendrimers. Whilst sixth generation dendrimers (192 primary amines) and higher have been prepared, for the present invention lower generation dendrimers are used, preferably G_0, G_1, G_2, G_3, G_4 or G_5 dendrimers (which have respectively 3, 6, 12, 24, 48 and 96 free amines). Preparation of PAMAM and related dendrimers is described by Tomalia et al. in Polymer Journal 17:117 (1985) and in U.S. Patent No. 4,587,329.

Preferably the starburst dendrimer-type backbone molecules are radially symmetrical with each optionally branched linker group being identical.

A branched linker group may be formed by the successive addition of linking moeities which may each be the same or different. A branched linker group

wherein the linking moieties are all identical is preferred. Alternatively, the branched linker group may be pre-formed and subsequently conjugated to the core moiety.

The core moiety may be any molecule to which a multiplicity of linker groups can be attached. Thus a G_0 core moiety can be expressed by the general formula

$B^{\circ}(L^{\circ}R^{\circ})_{n^{\circ}}$

where B° is a branching site, e.g. an optionally substituted nitrogen, phosphorus, silicon, boron or carbon atom or a homo- or heterocyclic ring, preferably having 5-8 ring members;
L° is a bond or a zero generation linking group, e.g. a C_{1-4} alkylene chain;
R° is a functional group capable of undergoing an addition, replacement or more preferably a condensation reaction whereby to conjugate at least one first-generation linker group L^1 to $B^{\circ}L^{\circ}$, e.g. an amine, hydroxyl or carboxyl group or a derivations thereof, e.g. an ester or amide;
and n° is an integer having a value of at least 2, preferably 3 or 4.

Using analogous terminology, an Xth generation, G_x, dendrimer backbone molecule would have the general formula.

$$B^{\circ}(L^{\circ}(L^{1}(L^{2}..(L^{x}R^{x}\quad)_{nx}..)_{n2}\quad)_{n1}\quad)_{n^{\circ}}$$

where R^x is a functional group capable of being conjugated to a chelant moiety, e.g. an amine group. For the PAMAM starburst dendrimers n° is 3, each of n^1, n^2, n^3 etc are 2, and R^x is an amine group.

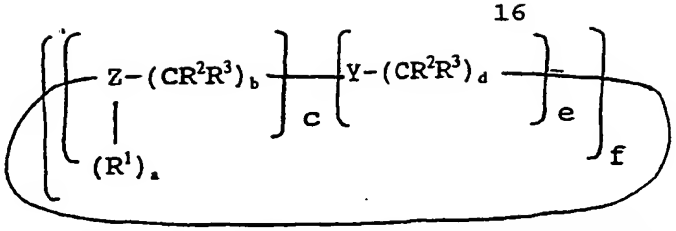
Suitable core G_o moieties thus include $N(CH_2CH_3COOCH_3)_3$, $(CH_3OOCCH_2CH_2)_2NCH_2CH_2N(CH_2CH_2COOCH_3)_2$, $(CH_3OOCCH_2CH_2)_2CHCH(CH_2CH_2COOCH_3)_2$, modifications thereto and derivatives thereof.

The preparation of a starburst dendrimer-type backbone molecule through four successive generations is described in Examples 10-14 hereunder.

The macrocyclic chelant moieties in the polychelants of this invention preferably derive from macrocyclic chelants which have a reactive carboxyl or amine group which is not essential for metal coordination bonding. The reactive group can be one of the groups which in the free chelant can function as a metal coordinating group so long as the conjugated chelant moiety retains the ability to complex metal ions. Alternatively the reactive group can be a substituent on a side chain of the chelant or on a backbone carbon.

More particularly, as used herein, a macrocyclic chelant is defined as a chelant having one continuous, linked, closed backbone consisting of donor atoms, such as for example N, P, B, O, S and As, spaced by carbon atoms e.g. carbons of optionally substituted methylene or cyclic, e.g. aromatic, groups or chains thereof, particularly preferably optionally substituted C_{2-4} alkylene chains. Any of the methylene groups or donor atoms, where permitted by valence conditions, can be substituted so long as the closed chain of the macrocycle remains intact.

In one preferred embodiment of the invention, the macrocyclic chelants are of formula III

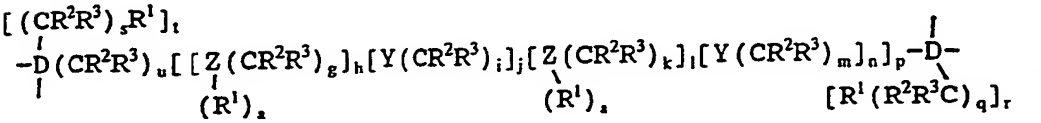


(III)

where a, b, d and e are independently zero or a positive integer, for b or d preferably 1, 2, 3 or 4; c and f are positive integers; the sum of all c's being at least 3, preferably 3, 4 or 5; the sum of b + d is at least 1; each Z is independently a nitrogen, oxygen, sulphur, phosphorus, boron or arsenic, preferably at least two, especially at least 3 of these being nitrogen; each Y is independently an optionally substituted 5 to 7 membered carbocyclic or heterocyclic ring;

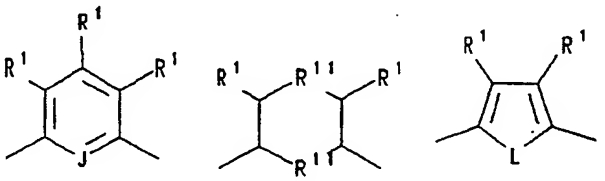
R^1 where present is independently hydrogen, optionally hydroxylated, optionally alkoxylated alkyl optionally carrying a group CO-G where G is OR^2 or NR^2_2 and where Z is phosphorus optionally also oxo, at least 3 $Z(R^1)_a$ moieties preferably having Z as nitrogen, a =1 and R^1 as an optionally substituted G-CO-alkyl group;

R^2 and R^3 which may be the same or different each independently is hydrogen, optionally alkoxylated, optionally hydroxylated alkyl, aryl, alkaryl or aralkyl or R^3 may also represent or be substituted by a group CO-G; and NR^2_2 may also represent a nitrogen-attached optionally substituted 5 to 7 membered heterocyclic ring optionally containing a further nitrogen oxygen, or sulphur ring heteroatom; and where in place of two CR^2R^3 groups, separated in either direction by at least one Z group, there may optionally be a bridging structure of formula



where u, g, h, i, j, k, l, m, n, q, r, s and t is each independently zero or a positive integer, for u, g, i, k and m preferably 1,2,3 or 4; p is a positive integer; h+l+j+n ≥ 1, preferably p(h+l) ≥ 1; and each D is independently boron, carbon, nitrogen, phosphorus or PO.

Preferred identities for the ring moieties Y include

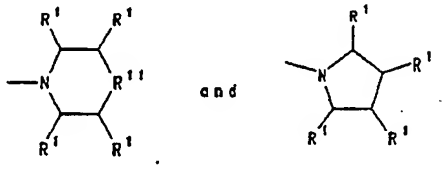


where J is CH, COH or N;

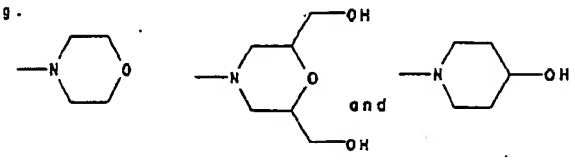
R^{11} is CH_2, CHOH, NR^1, O or S; and

L is O or S.

Preferred identities for the heterocyclic moieties NR^2_2 include



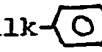
e.g.



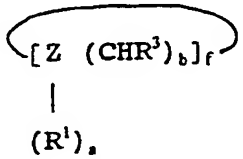
As indicated above, the macrocyclic chelant may include a second "cycle" which is created by linking the branches from two or more backbone atoms.

In the macrocyclic chelants, alkyl and alkylene

moieties, unless specified otherwise, preferably contain up to 8 carbon atoms, especially preferably up to 4 carbons. Hydroxy or alkoxy substituted moieties may be mono- or poly-substituted and substitution by both is contemplated. Any aryl moieties are preferably C_{6-10} carbocyclic or 5 or 6 membered heterocyclic rings. In the macrocycle, backbone heteroatoms , e.g. N,P,O and S are preferably separated by 1 to 8, especially preferably 2 to 6 carbon backbone atoms and, as mentioned, the macrocyclic chelant preferably contains at least 3 carboxyl groups or carboxyl derivative groups. Macrocyclic polychelants containing at least three ring nitrogen attached carboxyalkyl, especially carboxymethyl, groups are particularly preferred.

Linkage of the macrocyclic chelant to the backbone molecule may be effected through any reactive group, e.g. an R^1 or R^3 group, particularly preferably a CO-G group-containing R^1 group. Reaction of macrocycles with protonated ring heteroatoms (e.g. in DO3A) with Hal-CH_2CONH-alk-  -X"′ (where Hal is a halogen atom and alk and X"′ are as defined above) provides a reactive group for linkage to the dendrimeric backbone. Other standard coupling techniques can be used and thus the macrocyclic chelating moieties in the polychelants of the invention preferably comprise the residues of a chelant of formula III (i.e. groups of formula III but with one of the ring attached substituents modified or replaced to provide a link to the dendrimer).

Particularly preferred macrocyclic chelants include those of formula IV



(IV)

where each Z is N,O or S, preferably all or all but one Z being N;
each b is independently 2,3 or 4, preferably 2 or 3;
f is 3 or 4, preferably 4;
each R^1 is independently hydrogen, C_{1-3} alkyl or an optionally branched, optionally hydroxylated CO-G-alkyl group; and each R^3 is independently hydrogen or a hydroxyalkyl group.

Thus in particular, the macrocyclic chelants include the polyazacycloalkanepolycarboxylates, hexaazamacrocycles (HAMs) and cryptates including sepulchrates and sarcophagines.

Exemplary polyazacycloalkanepolycarboxylates include 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA), 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A), 1-oxa-4,7,10-triazacyclododecanetriacetic acid (DOXA), 1,4,7-triazacyclononanetriacetic acid (NOTA) and 1,4,8,11-tetraazacyclotetradecanetetraacetic acid (TETA). Additionally, the novel tetraazacycloalkanepolycarboxylates, DOTA-N(2-aminoethyl)amide and DOTA-N(4-aminophenethyl)amide are also contemplated.

The preparation of the tetraazacycloalkanepolycarboxylate ligands is well known. Synthesis of DOTA is described in U.S. Patent No. 4,647,447 (Gries et al.), U.S. Patent No. 4,639,365 (Sherry) and by Desreux et al. in Inorg. Chem. 19:1319 (1980). Additionally, DOTA is available commercially from Parrish Chemical Co., Orem, UT, USA. Preparation of DO3A is described in EP-A-292689 (Squibb). Desreux, Inorg. Chem., 19:1319 (1980); Bryden et al, Anal. Chem, 53:1418 (1981); Delgardo et al, Talanta, 29:816 (1982); Cacheris et al, Inorg. Chem, 26:958 (1987); Moi et al, Inorg. Chem, 26:3458 (1987) and Meares et al, Acc. Chem. Res., 17:202 (1984)

describe the properties and chemistry of the macrocyclic ligands DOTA, NOTA, TETA and their backbone-derivatized analogues, including the preparation of NOTA and TETA. U.S. Patent No. 4,678,667 (Meares et al.) teaches the preparation of a number of macrocyclic, side chain-derivatized ligands including DOTA and TETA. Derivatization of DOTA to form DOTA-N(2-aminoethyl)amide and DOTA-N(4-aminophenethyl)amide is described in detail hereinafter in Examples 2 and 3, respectively. The above cited references and all other references mentioned herein are hereby incorporated by reference in their entirety.

The hexaazamacrocycles include the series of N_6 macrocyclic chelates described in DeCola et al. in Inorg. Chem. 25:1729 (1986). That article also describes preparation of the HAMs and is incorporated herein by reference in its entirety.

Cryptates are polycyclic ligands which include sepulchrates, sarcophagines and macrocyclic polyethers (crown ethers) and macrobicyclic ligands. Preferred macrocyclic polyether cryptates include side-chain derivatized primary amine and carboxylate cryptates.

The sepulchrates include derivatives of the octaazamacrobicyclic system such as 1,3,6,8,10,13,16,19-octaazabicyclo[6,6,6]eicosane. Primary amine and carboxylate derivatives of these chelates are especially preferred. Synthesis of the chelates, as the cobalt complexes, is described in J. Amer. Chem. Soc. 104:6016 (1982). The sarcophagines include derivatives of the hexaazamacrobicyclic system such as 3,6,10,13,16,19-hexaazabicyclo[6,6,6]eicosane. Synthesis of sepulchrates and sarcophagines are described by Creaser et al. in J. Amer. Chem. Soc. 104:6016 (1982) and Geue et al. in J. Amer. Chem. Soc. 106:5478 (1984),

respectively. Izatt and Christensen, Eds., Synthetic Multidentate Compounds, Academic Press (1978) and Lehn et al, Acc. Chem. Res. 11:49 (1978) describe synthesis of cryptates. Cotton & Wilkinson "Advanced Inorganic Chemistry" describe a general method of crown ether template synthesis for preparing encapsulating nitrogen-containing macrocycles. Those references are incorporated herein by reference in their entirety.

Metals that can be incorporated, through chelation, include lanthanides and other metal ions, including isotopes and radioisotopes thereof, such as, for example, Mg, Ca, Sc, Ti, B, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Sr, Y, Zr, Tc, Ru, In, Hf, W, Re, Os, Pb and Bi. Particularly preferred radioisotopes of some of the foregoing include ^{153}Sm, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{89}Sr, ^{88}Y, ^{90}Y, ^{99m}Tc, ^{97}Ru, ^{103}Ru, ^{111}In, ^{186}Re, ^{188}Re, ^{203}Pb, ^{211}Bi, ^{212}Bi, ^{213}Bi, and ^{214}Bi. The choice of metal ion for chelation by polychelants of the invention will be determined by the desired therapeutic or diagnostic application.

The bifunctional polychelants of the invention involve coupling the magnifier to a site-directed molecule. The site-directed molecules may be any of the molecules that naturally concentrate in a selected target organ, tissue, cell or group of cells, or other location in a mammalian body, in vivo. These can include amino acids, oligopeptides (e.g. hexapeptides), molecular recognition units (MRU's), single chain antibodies (SCA's), proteins, Fab fragments, and antibodies. Examples of site-directed molecules include polysaccharides (e.g. CCK and hexapeptides), proteins (such as lectins, asialofetuin, polyclonal IgG, blood clotting proteins (e.g. hirudin), lipoproteins and glycoproteins), hormones, growth factors, and clotting factors (such as PF4). Exemplary site-directed proteins include polymerized fibrin fragments (e.g., E_1), serum

amyloid precursor (SAP) proteins, low density lipoprotein (LDL) precursors, serum albumin, surface proteins of intact red blood cells, receptor binding molecules such as estrogens, liver-specific proteins/polymers such as galactosyl-neoglycoalbumin (NGA) (see Vera et al. in Radiology 151: 191 (1984)) N-(2-hydroxy-propyl)methacrylamide (HMPA) copolymers with varying numbers of bound galactosamines (see Duncan et al., Biochim. Biophys. Acta 880:62 (1986)), and allyl and 6-aminohexyl glycosides (see Wong et al., Carbo. Res. 170:27 (1987)), and fibrinogen.

The site-directed protein can also be an antibody. The choice of antibody, particularly the antigen specificity of the antibody, will depend on the desired use of the conjugate. Monoclonal antibodies are preferred over polyclonal antibodies.

Human serum albumin (HSA) is a preferred protein for the study of the vascular system. HSA is available commercially from a number of sources including Sigma Chemical Co. Preparation of antibodies that react with a desired antigen is well known. Antibody preparations are available commercially from a variety of sources. Fibrin fragment E_1 can be prepared as described by Olexa et al. in J. Biol. Chem. 254:4925 (1979). Preparation of LDL precursors and SAP proteins is described by de Beer et al. in J. Immunol. Methods 50:17 (1982). The above described articles are incorporated herein by reference in their entirety.

In general, magnifiers are synthesized by conjugating the chelants to the backbone molecule prior to conjugating the backbone molecule to the site-directed macromolecule to produce a bifunctional polychelant. In most cases, the reaction conditions used for joining the chelants to the backbone molecule

would denature proteins. Therefore, to preserve its tertiary structure and biological function an antibody or other site-directed protein will not generally be conjugated to a backbone molecule before the chelant groups have been loaded onto that backbone molecule, unless of course this can be done without denaturing the protein. The metal ions can be added to form the metal complex of the polychelants prior to or following conjugation of the magnifier to the site-directed macromolecule. Preferably, the metal will be added prior to conjugation of the magnifier polychelant to most proteins, particularly antibodies, in particular to avoid adventitious binding of the metal to the protein. However, for some metal ions such as radionuclides with a short half-life, metallation will preferably be performed following conjugation, just prior to use.

In general, known methods can be used to join the macrocyclic chelants to backbone molecules. While for preferred macrocyclic chelants, such as DOTA, the conventional mixed anhydride and cyclic anhydride conjugation techniques are ineffective, it has been found that modifying the mixed anhydride procedure by reacting a polycarboxylic macrocyclic chelant in an anhydrous medium with an amine base of sufficient strength to abstract all the carboxyl protons (i.e. a high enough pKa) yields an amine salt which can react with an alkylhaloformate to produce an activated anhydride capable of conjugating to the backbone polyamine without causing the undesired cross-linking associated with prior art bifunctional polychelants. For most macrocyclic chelants tetramethylguanidine or an amine base of similar strength will be the preferred base.

More complex conjugation techniques, involving for

example the use of backbone derivatized macrocyclic chelants in a manner analogous to that of Meares et al. (supra), may of course be used but the increased cost and complexity of the overall production makes this a less desirable route. Similarly the chelants can be attached to the backbone polymer by a haloacetylhalide, a phosgene or a thiophosgene method depending on the available reactive group on the chelating agent.

For macrocycles with a pendant carboxylate, including but not limited to DOTA, TETA, TRITA (1,4,7,10-tetraazacyclotridecanetetraacetic acid) and NOTA, one of the carboxylates can form an entity which can react with a primary amine group of the backbone polymer. Methods of forming a reactive entity from a carboxylate group include the modified mixed anhydride reaction for example using isobutylchloroformate (IBCF), or the formation of an "activated ester" using a carbodiimide (DCC or EDAC, cf. Pierce Catalog (1988), pages 252 and 253). Both reaction sequences give rise to a backbone polymer multiply substituted with the macrocyclic chelant moieties through stable amide linkages. The modified mixed anhydride method however is the preferred method for use in joining the carboxylate-containing macrocyclic chelants to the backbone polymer.

The modified mixed anhydride reaction is performed in an anhydrous solvent preferably with a melting point below 5°C, cooled to a temperature not lower than 5°C or greater than about 55°C above its freezing point. The solubilization of the chelant in the appropriate solvent is conveniently effected by preparation of the amine salt of the chelant using the amine base in situ.

The choice of base is determined by the pKa of the relevant carboxylates. For most macrocycles,

tetramethylguanidine (TMG) is especially preferred. In general, bases will conveniently be selected from those bases whose pKa value exceeds the highest pKa of the macrocyclic chelant by at least 0.5, preferably 0.8, especially preferably at least 1.0. Amine bases having pKa's of at least 11, especially at least 11.3, particularly at least 12, are particularly preferred and besides TMG particular mention may be made of piperidine, quinuclidine and N-ethylpiperidine and more especially DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and DBN (1,5-diazabicyclo[4.3.0]non-5-ene). Further bases are listed by Martell and Smith in "Critical Stability Constants" Vol. 5, first supplement, Plenum Press, NY 1982.

The appropriate quantity of neat (chilled) alkylhaloformate is now added with stirring and the original temperature of the solvent is maintained by cooling, e.g. by addition of coolant, if required. Isobutylchloroformate is especially preferred. The resulting activated anhydride of the macrocyclic chelant can be reacted with an amine-containing dendrimer to form a magnifier polychelant. The magnifier polychelant, for most applications, is metallated at this point and purified by chromatography or crystallization to remove excess metal ions and lower molecular weight metal complexes. For use with target-specific molecules the magnifier polychelant, or the at least partially metallated form thereof, still containing at least one free amine, is conjugated to the targetting molecule, for example by reaction with one of many well-known heterobifunctional coupling agents. In situations where prior metallation is not appropriate, e.g. with radionuclide metal ions with short half-lives, the bifunctional polychelant can be prepared using a metal-free magnifier and coupling as described above, followed by metallation (vide infra) and final rapid,

simple purification by chromatography or filtration.

The macrocyclic chelants can also be linked to the backbone polymer through a non-coordinating primary amine group. Macrocyclic chelants having a non-coordinating primary amine group include primary amine side-chain-derivatized DOTA macrocycles, primary amine-derivatized DO3A, and primary amine-derivatized hexaaza and octaaza macrocycles and macrobicycles (the HAMs, sepulchrates and sarcophagines) as well as the broad class of derivatized crown ether cryptates.

The non-coordinating primary amine group on these chelants can be reacted with a haloacetylhalide under well-known conditions to form a haloacetamide. The haloacetamide can react with a primary amine of the backbone polymer to form a stable amide linkage between the chelant and the polymer. The haloacetylhalide method described in De Riemer et al, J. Labelled Compd. Radiopharm. 18:1517 (1981) can be used to join amine-containing chelants to the backbone polymer.

Amine groups on a macrocyclic chelant can also be reacted with phosgene to generate a reactive isocyanate group, or with thiophosgene to generate a reactive isothiocyanate group. Those groups can react with a primary amine of the backbone polymer to form a stable urea or more stable thiourea linkage, respectively, between the ligand and the backbone polymer. Gansow, Inorg. Chimica Acta 91:213 (1984) and Moi et al, J. Amer. Chem. Soc. 110:6266 (1988) describe methods of linking chelants to proteins having an amine group through formation of the isocyanate or isothiocyanate moieties using the phosgene or thiophosgene methods, respectively. See also Desreux, Inorg. Chem. 19:1319 (1980); Bryden et al, Anal. Chem 53:1418 (1981); Delgardo et al, Talanta 29:815 (1982); Cacheris et al,

Inorg. Chem. 26:958 (1987); Mōi et al, Inorg. Chem 26:3458 (1987) and Meares et al, Acc. Chem. Res. 17:202 (1984).

As indicated earlier the choice of metal ions to be chelated by the polychelants of the invention depends upon the diagnostic or therapeutic technique for which the resulting polychelate is to be used. For MRI, the metal ions should be paramagnetic, and preferably non-radioactive. For X-ray and ultrasound imaging, heavy metal ions, e.g. with atomic numbers of at least 37, preferably at least 50, should be used, again preferably non-radioactive species. For scintigraphy or radiotherapy the metal ions should of course be ions of radioactive isotopes.

Methods of complexing metal ions with chelants and polychelants are within the level of skill in the art. Each of the metals used can be incorporated into a macrocyclic chelant moiety by one of three general methods: direct incorporation, template synthesis and/or transmetallation. Direct incorporation is preferred.

The metal ions Fe(III), Cr(III), Mn(II), Hg(II), Pb(II), Bi(III) and the lanthanides can be directly incorporated into polyaminopolycarboxylates by the following general procedure. A water-soluble form of the metal, generally an inorganic salt, is dissolved in an appropriate volume of distilled, deionized water. The pH of the solution will be below 7. An aqueous solution containing an equimolar amount of the polychelant is added to the metal solution at room temperature while stirring. The pH of the mixture is raised slowly by addition of base, typically 0.1 M NaOH, until the donor groups of the polychelant are deprotonated, generally in the pH range of 7 to 9,

depending on the chelant moieties. Particular care must be taken with the lanthanide ions to maintain the pH below 8 to avoid precipitation of the metal hydroxide. Metal incorporation into DOTA derived and related macrocylic chelant moieties will normally be a slow process, as described in the references cited below. Specific examples of the procedure are contained in the Examples hereto and in the following references.

Choppin et al, J. Inorg. Nucl. Chem., 33:127 (1971), Margerum, Rec. Chem. Prog., 24:237 (1973) and D'Olieslager et al, J. Inorg. Nucl. Chem., 35:4255 (1973) describe direct incorporation of the lanthanides into polyaminopolycarboxylates. Margerstadt, Mag. Res. Med., 3:808 (1986) and WO-A-87/06229 describe incorporation of Gd(III) into DOTA. A method of preparing Bi and Pb complexes of DOTA is described by Kumar et al, J. Chem. Soc. Chem. Commun., 3:145 (1989). The above references are incorporated herein by reference in their entirety.

Direct incorporation of Hf, Zr, W, Hg and Ta can be performed according to well known methods. See, for example, U.S. Patent No. 4,176,173 (Winchell).

Transmetallation is useful when the metal ion needs to be reduced to a more appropriate oxidation state for the donor atoms of the chelant moiety to bind. For example, to incorporate ^{99m}Tc or $^{186/188}Re$, the metal ion must be reduced to Tc(V) or Re(V) by the use of reducing agents such as $SnCl_2$ or cysteine by well known methods. This method requires formation of an intermediate complex. A typical example is the reduction of ^{99m}Tc with Sn in the presence of a weakly coordinating ligand such as glucoheptonate prior to complexation with chelants such as DOTA. These methods are well known in the radiopharmaceutical art. ^{67}Cu utilizes tetraamine

chelates such as tet A or tet B (see Bhardaredj et al., JACS, 108:1351 (1986)) to stabilize Cu(II) for reaction with stronger-binding chelants.

Template synthesis can be performed by the method described by Smith et al. in Inorg. Chem., 24:3469 (1985) and 27:4154 (1988). In the case of the HAM systems, the metal ion is incorporated into the macrocyclic chelant by building the chelant around the metal ion via template synthesis. Well-known template synthesis methods are described by Smith et al. (Supra) for lanthanide template syntheses. The sepulchrate and sarcophagine macrobicyclic chelants may be similarly prepared by a template synthesis around Co. The Co is removed by reduction to Co(II) and extraction with 15 M HBr. The metal-free chelant may then be metallated via reaction with a simple metal salt by refluxing in methanol, or by transmetallation from a donor complex such as glucoheptonate, ascorbate, acetate or citrate salts. Use of triflate and/or perchlorate salts are preferred.

The broad class of crown ethers and cryptates, especially those containing N, O, and S, can be metallated in a similar fashion using one or more of the methods described above.

Methods for attaching backbone polymers to antibodies and other proteins are within the level of skill in the art. Such methods are described in Pierce 1989 Handbook and General Catalog and the references cited therein, Blatter et al, Biochem., 24:1517 (1985) and Jue et al, Biochem., 17:5399 (1978). The references cited above are incorporated herein by reference in their entirety.

The metal chelates of the polychelants of the

invention, especially the bifunctional polychelants but optionally also the magnifier polychelants, may be administered to patients for imaging in amounts sufficient to yield the desired contrast with the particular imaging technique. Generally dosages of from 0.001 to 5.0 mmoles of chelated imaging metal ion per kilogram of patient bodyweight are effective to achieve adequate contrast enhancements. For most MRI applications preferred dosages of imaging metal ion will be in the range from 0.02 to 1.2 mmoles/kg bodyweight while for X-ray applications dosages of from 0.5 to 1.5 mmoles/kg are generally effective to achieve X-ray attenuation. Preferred dosages for most X-ray applications are from 0.8 to 1.2 mmoles of the lanthanide or heavy metal/kg bodyweight.

Where a contrast agent is required to collect in the blood pool or in the extracellular fluid, then preferably a low generation dendrimeric backbone will be used, for example a fourth or fifth generation dendrimeric backbone. Such polychelants have enhanced relaxivity compared to known blood pooling and ECF contrast agents and thus a lower effective dosage can be administered.

For X-ray applications, to extend the photon energy range over which the polychelates of the invention are optimally effective the polychelates used may be of two or more different metals, either as mixtures of homopolychelates or as a heteropolychelate.

The compounds of the present invention may be formulated with conventional pharmaceutical or veterinary aids, for example emulsifiers, fatty acid esters, gelling agents, stabilizers, antioxidants, osmolality adjusting agents, buffers, pH adjusting agents, etc., and may be in a form suitable for

parenteral or enteral administration, for example injection or infusion or administration directly into a body cavity having an external escape duct, for example the gastrointestinal tract, the bladder or the uterus. Thus the compounds of the present invention may be in conventional pharmaceutical administration forms such as tablets, capsules, powders, solutions, suspensions, dispersions, syrups, suppositories etc.; however, solutions, suspensions and dispersions in physiologically acceptable carrier media, for example water for injections, will generally be preferred.

The compounds according to the invention may therefore be formulated for administration using physiologically acceptable carriers or excipients in a manner fully within the skill of the art. For example, the compounds, optionally with the addition of pharmaceutically acceptable excipients, may be suspended or dissolved in an aqueous medium, with the resulting solution or suspension then being sterilized. Suitable additives include, for example, physiologically biocompatible buffers (as for example, tromethamine hydrochloride), additions (e.g., 0.01 to 10 mole percent) of chelants (such as, for example, DTPA, DTPA-bisamide or non-complexed magnifier polychelant) or calcium chelate complexes (as for example calcium DTPA, CaNaDTPA-bisamide, calcium-magnifier polychelant or CaNa salts of magnifier polychelants), or, optionally, additions (e.g., 1 to 50 mole percent) of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate combined with metal chelate complexes of magnifier ligands, and the like).

If the compounds are to be formulated in suspension form, e.g., in water or physiological saline for oral administration, a small amount of soluble chelate may be

mixed with one or more of the inactive ingredients traditionally present in oral solutions and/or surfactants and/or aromatics for flavoring.

For MRI and for X-ray imaging of some portions of the body the most preferred mode for administering metal chelates as contrast agents is parenteral, e.g., intravenous administration. Parenterally administrable forms, e.g., intravenous solutions, should be sterile and free from physiologically unacceptable agents, and should have low osmolality to minimize irritation or other adverse effects upon administration, and thus the contrast medium should preferably be isotonic or slightly hypertonic. Suitable vehicles include aqueous vehicles customarily used for administering parenteral solutions such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection and other solutions such as are described in Remington's Pharmaceutical Sciences, 15th ed., Easton: Mack Publishing Co., pp. 1405-1412 and 1461-1487 (1975) and The National Formulary XIV, 14th ed. Washington: American Pharmaceutical Association (1975). The solutions can contain preservatives, antimicrobial agents, buffers and antioxidants conventionally used for parenteral solutions, excipients and other additives which are compatible with the chelates and which will not interfere with the manufacture, storage or use of products.

Viewed from a further aspect the invention provides an image enhancing or therapeutic composition comprising a metal chelate of a polychelant of the invention or a salt thereof together with at least one pharmaceutical carrier or excipient.

Viewed from a still further aspect the invention

provides the use of a polychelant according to the invention or a chelate or salt thereof for the manufacture of an image enhancing contrast medium or a therapeutic composition.

Viewed from another aspect the invention provides a method of generating an image of a human or non-human animal, especially mammalian, body which method comprises administering to said body an image enhancing amount of a polychelate according to the invention or a salt thereof and thereafter generating an image e.g. an MR, X-ray, ultrasound or scintigraphic image, of at least a part of said body.

Viewed from a still further aspect the invention provides a method of radiotherapy of the human or animal body said method comprising administering to said body a therapeutically effective amount of a radioactive metal chelate of a polychelant according to the invention.

Viewed from a yet still further aspect the invention provides a method of producing a polychelant according to the invention or a chelate thereof, said method comprising conjugating a plurality of macrocyclic chelants to an up to fifth generation dendrimeric polymer, e.g. a polyamine, optionally metallating the resulting polychelant, and optionally conjugating the polychelant or the chelate thereof to a site-specific molecule.

Viewed from another aspect the invention provides a detoxification composition comprising a polychelant according to the invention or a weak chelate complex or salt thereof with physiologically tolerable counterions, together with a pharmaceutical carrier or excipient.

Viewed from a still further aspect, the invention

provides a method of metal detoxification comprising administering to a human or non-human animal a detoxifying amount of a polychelant according to the invention or a weak chelate complex or salt thereof with physiologically tolerable counterions.

This invention is further illustrated by the following specific but non-limiting examples. Temperatures are given in degrees Celsius and concentrations as weight percentages unless otherwise specified.

EXAMPLE 1

Preparation of DOTA Carboxycarbonic Anhydride

DOTA(0.808 g, 2.0 mmol) was suspended in 5.0 ml of anhydrous acetonitrile. Tetramethylguanidine (1.00 ml, 8.0 mmol) was added and the mixture stirred under an atmosphere of nitrogen for about 5 minutes at ambient temperature until the DOTA was dissolved. The resulting solution was cooled to -25°C under an atmosphere of nitrogen and stirred while adding 0.260 ml (2.0 mmol) of isobutylchloroformate (IBCF), slowly over 5 minutes. The resulting slurry was stirred for 1 hour at -25 C.

EXAMPLE 2

Preparation of DOTA-N(2-aminoethyl)amide

To the cold slurry from Example 1 was added a solution of mono-BOC-ethylenediamine (0.320g, 2mmol) in 2 ml acetonitrile and the mixture stirred 6 to 12 hours at ambient temperature. The mixture was brought to 20 ml with H_2O, treated with 6 ml of concentrated HCl, and then stirred overnight to effect removal of the protecting group. The solution was evaporated to

dryness. The residue was purified by ion exchange chromatography on DOWEX AGI-X8 resin. Evaporation of the appropriate fractions afforded 0.35g of a semi-crystalline glass. ^{1}H NMR demonstrated the expected product, as well as some residual acetate (from chromatography).

EXAMPLE 3

Preparation of DOTA-N(4-aminophenethyl)amide

To the cold slurry from Example 1 is added a solution of 4-nitrophenethylamine (0.332g, 2mmol) in 4.0 ml acetonitrile. The mixture is stirred 6 to 12 hours at ambient temperature. After evaporation to dryness, the residue is redissolved in water and pH adjusted to 10.5 with NaOH to form a mixture which is extracted with ethyl acetate to remove unreacted amine. The product, DOTA-N-(4'-nitrophenethyl)amide, is isolated by ion exchange chromatography on DOWEX AGI-X8 resin. Following evaporation of the appropriate fractions, the residue is dissolved in water in a Parr reactor, and 0.1 g of 5% palladium on activated carbon is added to form a reaction mixture. The reaction mixture is hydrogenated at 30-40 psi until the pressure ceases to drop. The product is isolated by filtering off catalyst and evaporating the filtrate to dryness.

EXAMPLE 4

Activation of Amino Group of DOTA-N(2-aminoethyl)amide with Thiophosgene - Conversion to Isothiocyanate Groups

An aqueous solution of the product prepared in Example 2 is added to an equal volume of chloroform containing thiophosgene and sodium bicarbonate, each of which is in four-fold molar excess with respect to the

target amino group. The mixture is stirred vigorously for 1-2 hours, and the phases are separated. The aqueous phase is washed with chloroform, and then it is evaporated to dryness. The resultant solid product is washed with ethanol and dried in vacuo.

The procedure is repeated, substituting the product of Example 3 for the product of Example 2.

EXAMPLE 5

Activation of Amino Group of DOTA-N(2-Aminoethyl)Amide with Bromoacetyl Chloride - Conversion to Bromoacetamide Groups

An aqueous solution of the product prepared in Example 2 (20mg/ml) which also contains triethylamine (20mg/ml) is treated with an equal volume of a chloroform solution of bromoacetyl chloride (30mg/ml), and the two-phase mixture is stirred vigorously for 1-2 hours. Water is added, to double the volume of the aqueous phase, and the mixture is extracted with ethyl acetate. The aqueous phase is evaporated to dryness and the residue triturated with acetone and dried in vacuo.

The procedure is repeated, substituting the product of Example 3 for the product of Example 2.

EXAMPLE 6

Preparation of DOXA and DOXA carboxycarbonic ahydride

(a) Synthesis of 1-oxa-4,7,10-triazacyclododecane-4,7,10-triacetic acid (DOXA)

This compound was prepared as described by Amorim M.T.S. et al. in Talanta 35(9): 741-745 (1988).

(b) DOXA carboxycarbonic anhydride

DOXA (4 mmol, 1.38 g) is suspended in 7.0 ml of acetonitrile. Tetramethylguanidine (TMG, 12 mmol, 1.38 g, 1.5 mL) is added and the mixture refluxed until homogeneous. The resulting solution is cooled to -30°C under an atmosphere of nitrogen and stirred while adding isobutylchloroformate (4 mmol, 0.520 mL) slowly over 5 min. The resulting slurry is stirred for 1 hour at -30°C.

EXAMPLE 7

Preparation of TETA and TETA carboxycarbonic anhydride

(a) Synthesis of 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid (TETA)

This material was prepared according to the method of Delgado et al., Talanta 29: 815-822 (1982).

(b) TETA carboxycarbonic anhydride

TETA (4.4 mmol, 1.9 g) was suspended in 10 mL of acetonitrile. Tetramethylguanidine (TMG, 17.6 mmol, 2.2 mL) was added and the slurry refluxed for 1 hour. The resulting solution was cooled to ambient temperature and dried over 4 Å molecular sieves for 4-8 hours. The solution was decanted from the sieves and cooled to -30°C under an atmosphere of nitrogen, then stirred while adding 4.4 mmol, 0.510 mL of isobutyl-chloroformate slowly over 5 min. The resulting mixture was stirred 1 hour at -30°C and allowed to warm to -10°C.

EXAMPLE 8

Preparation of Core Adduct for PAMAM backbone polymer

6.6g gaseous NH_3 was dissolved in 100g methanol at 4°C in approximately 30 minutes with stirring. The gas flow was monitored using bubbles in hood. The resultant solution was added dropwise to 195g methyl acrylate over 3 hours 10 minutes. The maximum temperature observed was 30°C and the mixture was stirred for 3 days. Residual methyl acrylate and methanol were removed using rotary evaporation (vacuum pump at approximately 5mmHg, 38°C for 1 hour) to yield 170g of a clear, oily viscous solution ^1H NMR demonstrated that there was no residual methyl acrylate in the end-product.

$$NH_3(g) + 3CH_2{=}CHCOOCH_3 \xrightarrow{MeOH} N(CH_2CH_2COOCH_3)_3$$

EXAMPLE 9

Preparation of first generation ($G_{1.0}$) PAMAM Derivative

29g of the triester formed in Example 8 was combined slowly with 500g ethylenediamine in 215g MeOH. The mixture was allowed to stand for over 60 hours. Excess methanol and ethylenediamine were removed using rotary evaporation (60°C, 1-2mmHg).
32.6g of pale yellow thick syrup was obtained.
^1H NMR was used to confirm the identity of the reaction product.

EXAMPLE 10

Preparation of second generation $G_{2.0}$ PAMAM Derivative

a) $G_{1.5}$ PAMAM Derivative

174g methylacrylate was heated to 32°C. 32.6g of the product from Example 9 was dissolved in 100g methanol and this was added using a dropping funnel over 1½ hours to the methylacrylate with stirring under reflux. The mixture was stirred for a further 6 hours at 32°C, and then cooled to ambient temperatures and allowed to stand for 20 hours. Residual methanol/methylacrylate were removed by using a rotary vacuum pump at 50°C. ^{1}H NMR confirmed the absence of methylacrylate. Theoretical yield 79.7g. Obtained 78g (98% of theory).

b) $G_{2.0}$ PAMAM Derivative

700ml ethylenediamine were combined with 420g methanol at ambient temperature. The mixture was heated by exothermic reaction, but was cooled to 40°C after 1 hour. 78g of the $G_{1.5}$ derivative from (a) was mixed into 100g methanol to produce a bright yellow solution and was added to the ethylenediame/methanol mixture over 10 minutes with stirring. The temperature increased during addition to 50°C. The mixture was allowed to stand for 3 days after which rotary evaporation was used to remove any excess ethylenediamine and methanol (5mmHg).

Theoretical yield 91.8g. Obtained 92g.

EXAMPLE 11

Preparation of third generation ($G_{3.0}$) PAMAM Derivative

a) $G_{2.5}$ PAMAM Derivative

200g methylacrylate in a 2 litre round bottomed flask was placed in a flask equipped with a stirrer and condenser. 92g of the product obtained from Example 10 dissolved in 300ml MeOH was added over 2 hours. During addition, the temperature rose from 20°C to 35°C. The

mixture was stirred at ambient temperature for 2 hours. Residual methylacrylate and methanol were removed by rotary evaporation. ^1H NMR was used to confirm the purity of the product.

	C	H	N
Calc:	53.78	8.02	12.83
Found:	52.67	8.01	10.32

b) $G_{3.0}$ PAMAM Derivative

1400ml ethylenediamine was conbined with 700mls methanol in a 3 litre flask equipped with a stirrer, thermometer and condenser. The mixture heated exothermically to 70°C, and dropped to 40°C after ¼ hour. 124g of the product from (a) was dissolved in 250mls methanol, stirred until completely solubilised and added dropwise at ambient temperatuure over 40 hours, to the ethylenediamine/methanol mixture. After being stirred for 24 hours, excess methanol and ethylenediamine was removed by rotary evaporation, 70°C. 180g of a yellow oil/gum was obtained.

EXAMPLE 12

Preparation of fourth generation ($G_{4.0}$) PAMAM Derivative

a) $G_{3.5}$ PAMAM Derivative

92g of the end-product from Example 11 was dissolved in methanol and was added slowly over 2 hours to a stirred solution and methylacrylate present in 40-fold excess (140g) at ambient temperature. The reaction mixture was left for 48 hours, with stirring. Rotary evaporation was used to remove any excess methylacrylate and methanol. 160g of a golden yellow residue (94% of theory) was obtained.

b) $G_{4.0}$ PAMAM Derivative

1600ml ethylenediamine was added to 700mls methanol with stirring. 100g of the product from (a) in 500ml methanol was added slowly thereto over 72 hours and was then stirred at ambient temperature for approximately 100 hours. Ethylenediamine was removed using a rotary evaporator. n-Butanol was added to azeotrope off any remaining ethylene. Excess n-butanol was removed by rotary evaporation.

EXAMPLE 13

Preparation of $G_{2.0}$ PAMAM - poly DOTA

The $G_{2.0}$ PAMAM dendrimer prepared in Example 10 (10g, 0.01 mol) is combined with 12 equivalents of DOTA carboxycarbonic anhydride (0.13 mol) prepared as in Example 1, by slowly mixing a precooled (0° C) acetonitrile solution (20 ml) of dendrimer to the DOTA mixed anhydride slurry over 10 minutes and gradually allowing the reaction mixture to warm to ambient

temperature. The reaction mixture is worked up and purified according to the procedure developed for polylysine described in Sieving et al., Bioconj. Chem. 1(1):65-71 (1990).

EXAMPLE 14

Activation of Human Serum Albumin (HSA)

HSA contains one native thiolate group. This group was blocked by alkylation as described below. 50 ml of 0.05 M Tris-HCl, pH 7.3 was adjusted to pH 8.0 using 1.0 M Tris base. HSA (1g, 15 μmol) was added to the solution. After stirring until homogeneous, the flask containing the solution was purged with dry nitrogen, sealed with a septum and wrapped in aluminum foil to exclude light.

A solution of iodoacetamide (15 mg, 80 μmol) in 4.0 ml of 1 N NaOH was added dropwise by using a syringe inserted through the septum. The resulting reaction mixture was stirred for 45 minutes at ambient temperature in the dark. The reaction mixture was dialyzed against 3.5 liters of 0.05 M sodium bicarbonate, pH 8.0, for 12 hours, with a buffer change at 6 hours. The dialysate was lyophilized to dryness to form a white fibrous mass.

The absence of free thiols in the preparation was demonstrated by the method of Ellman (see Arch. Biochem. Biophys. 74: 443 (1958)). The purity of the preparation was determined by measuring the specific absorbance of a 1 mg/ml solution of the product at 280 nm (1cm path). The analysis showed that a purity of 99% with yield of 0.903 g was obtained.

100 mg of the above thiol-blocked HSA was dissolved in 50 ml of 60 mM triethanolamine, 7 mM monopotassium phosphate, 100 mM NaCl, 1 mM EDTA, pH 8.0. The solution was degassed for 10 minutes by stirring under vacuum,

then covered with an atmosphere of nitrogen in a septum-sealed flask. After cooling the flask in an icebath, a solution of 2-iminothiolane (8.5 mg) in 100 µl of 1 M triethanolamine, pH 8.0 was added to the flask by syringe. The mixture was stirred for about 90 minutes at 0-4°C. After overnight dialysis against 3.5 liters of 0.08 M sodium phosphate, 0.5 mg/ml; EDTA, pH 8.0 with frequent buffer changes, spectrophotometric analysis by the method of Ellman demonstrated the presence of 2.7 thiols per mole of HSA.

Activation of Gadolinium Polychelates for Coupling to HSA

A sample of the polychelate is dissolved in Na_2HPO_4, pH 8. A solution of succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) in DMSO is added dropwise to form a mixture. The mixture is stirred for 30 minutes at ambient temperature to form a solution. The resulting solution is dialyzed for 12 hours against H_2O with a single change at 6 hours to remove excess SMCC.

Coupling of Gadolinium Polychelates to HSA

The solutions prepared as described above are combined and stirred for 4 hours to form a mixture. The mixture is lyophilized. The resultant solid is dissolved in H_2O and dialyzed for 6 hours against H_2O. The dialysate is chromatographed on Sephacryl S-300. The fractions with significant absorbance at 280 nm are pooled and lyophylized. A sample of this solid is dissolved in water (1 mg/ml) and assayed for HSA (using a spectrophotometer and measuring absorbance at 280 nm) and Gd (using directly coupled plasma atomic absorption (DCP-AA)) to determine the number of metal ions bound per mole HSA.

EXAMPLE 15

Activation of Antibody L6

Antibody L6 (5 mg, 33 nmol) in 2.5 mL 60 mM triethanolamine, 7 mM monopotassium phosphate, 100 mM NaCl, 1 mM EDTA, pH 8, was degassed for 10 min by stirring under vacuum and then put under an atmosphere of N_2. After cooling for 30 min in an icebath, 70 µL of 2-iminothiolane.HCl (2 µmol) in the same triethanolamine buffer was added. The mixture was stirred for 90 min at 0-4°C. The resulting mixture was then transferred into 150 mM NaCl, 80 mM sodium phosphate, 0.5 mg/ml EDTA pH 8, and concentrated by ultrafiltration. The concentrated antibody was diluted to 2.5 mL with the same buffer. Spectrophotometric analysis by Ellman's method demonstrated the presence of 2.2 thiols per molecule of antibody.

Activation of Gadolinium Polychelates for coupling to Antibody L6

A sample of the polychelate is dissolved in 0.008M Na_2HPO_4, pH 8. A solution of SMCC in DMSO is then added dropwise. The mixture is stirred for 30 min while protected from light. Excess SMCC and buffer salts are then removed by ultrafiltration using an Amicon centricon 30 microconcentrator (5000 rpm for 45 min, then repeated twice with H_2O added to the concentrated polychelate). The polychelate is then diluted with deionized H_2O. Reaction of an aliquot of this polychelate with a known amount of 2-mercaptoethanol and measurement of the residual sulfhydryls by Ellman's method allows the number of maleimide residues per molecule of polychelate to be estimated.

Coupling of Gadolinium Polychelates to Antibody L6

The solutions prepared as described above are combined and stirred overnight. The mixture is concentrated by ultrafiltration and then chromatographed on Sephacryl S-300. The fractions absorbing significantly at 280 nm are pooled and concentrated to a known volume.

EXAMPLE 16

Preparation of $ClCH_2CONHCH_2C_6H_4pNO_2$

A methylene chloride solution of triethylamine (3 mL, 30 mmol) and 4-nitrobenzylamine (1.74 g, 9.2 mmol) was added at 0°C to a cooled solution of chloroacetylchloride (0.73 mL, 9.2 mmol) and the resulting solution allowed to warm to ambient temperature. After stirring for 18 hours, the reaction mixture was extracted with 0.1 N HCl and also saturated sodium bicarbonate. The organic layer was dried over sodium sulphate. The volatiles were removed under pressure, and the crude product taken up in chloroform and the product purified by flash chromatography (5:1 $CHCl_3/CH_3OH$) and isolated as a pale yellow solid by removal of volatiles in vacuo.

EXAMPLE 17

Preparation of DOTA-$G_{3.0}$-Dendrimer magnifier

An acetonitrile solution of tris-t-butyl-DO3A and $ClCH_2CONHCH_2(C_6H_4)pNO_2$ (Example 16) are heated at 65°C for 24 hours. The chelant-linker product is isolated by toluene work-up followed by chromatography. The resultant product is dissolved in an appropriate solvent and, in a Parr reactor, is hydrogenated with 5% palladium on activated carbon. The product 4-aminobenzylamide-chelant is isolated by filtration and

by subsequent removal of volatiles. This product is treated with thiophosgene to convert the 4-amino group to an isothiocyanate.

A methanol solution of the chelant-isothiocyanate linker is added to a methanol solution of third generation ($G_{3.0}$) PAMAM dendrimer (Example 11b). The reaction mixture is stirred for 48 hours with the reaction progress being followed by FT-IR. The product is purified and isolated using size-exclusion chromatography.

EXAMPLE 18

Activation of Asialoorosomucoid glycoprotein (ASGP)

ASGP, a liver specific protein, contains no native sulphydryl residues and the activation may be carried out directly. A solution of ASGP (26.4 mg, 0.6 micromol) in Ellman's buffer (15 mL, pH 8.36) was degassed under vacuum and cooled to 0°C under a nitrogen atmosphere. A solution of 2-iminothiolane hydrochloride (13.4 mg, 0.097 mmol) in triethanolamine hydrochloride buffer (1 mM, pH 8.42, 0.6 mL) was added and the mixture was stirred at 0°C for 2 hours. The solution was then dialyzed against phosphate buffer (0.08 mM phosphate containing 0.5 weight % EDTA, pH 7.8, 600 mL) overnight. The protein content in the dialysate was assayed by measurement of absorbance at 280 nm. The thiol content was assayed by Ellman's method. The number of thiol groups per ASGP was assayed to be 1.5.

Activation of gadolinium polychelates for coupling to activated ASGP and coupling to the activated ASGP

Activation and coupling are performed analogously to Example 15.

CLAIMS:

1. A polychelant comprising an up to fifth generation dendrimeric backbone moiety with linked thereto a plurality of macrocyclic chelant moieties capable of complexing metal ions, and metal chelates and salts thereof.

2. A compound according to claim 1 of formula I

B(L)n (I)

where B is a said dendrimeric backbone moiety, n is an integer having a value of at least 3, and each L is independently the residue of a macrocyclic chelant, or a chelate or salt thereof.

3. A compound according to claim 2 wherein B is the residue of a starburst dendrimer.

4. A compound according to either of claims 2 and 3 wherein n is 6 to 96.

5. A compound according to any one of claims 1 to 4 wherein said backbone moiety is an up to fourth generation dendrimeric moiety.

6. A compound according to any one of claims 1 to 5 wherein the macrocyclic chelant moieties are linked to said backbone moiety via amide groups.

7. A compound according to any one of claims 1 to 6 wherein at least some of said chelant moieties are unmetallated.

8. A compound according to any one of claims 1 to 7 wherein at least some of said chelant moieties are metallated by metal ions selected from the group consisting of the paramagnetic ions of Fe, Mn, Co, the

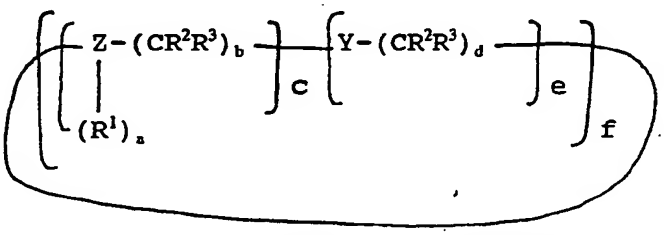
ions of Bi, Hg, Os, Ph, Zr, and lanthanides and the radioactive ions of In, Tc, Y, Re, Pb, Cu, Ga, Bi and Sm.

9. A compound according to any one of claims 1 to 8 wherein said backbone moiety is a polyaminoamido polymer or a derivative thereof.

10. A compound according to claim 9 wherein said backbone moiety is a first, second, third or fourth generation polyaminoamido starburst dendrimer polymer.

11. A compound according to claim 9 wherein said backbone moiety is a fifth generation polyaminoamido starburst dendrimer polymer.

12. A compound according to any one of claims 1 to 11 wherein said macrocyclic chelant moieties are residues of macrocyclic chelants of formula III



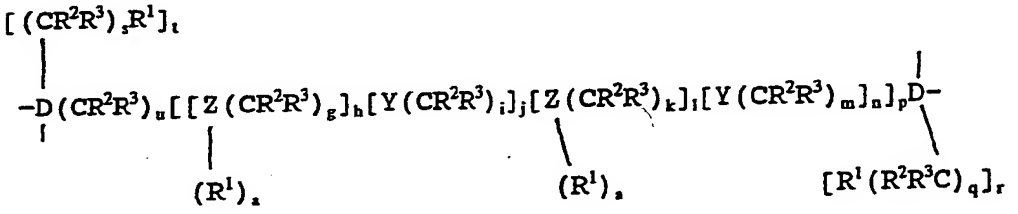
(III)

where a, b , d and e are independently zero or a positive integer; c and f are positive integers, the sum of all cs being at least 3; the sum of b + d is at least 1; each Z is independently a nitrogen, oxygen, sulphur, phosphorus, boron or arsenic; each Y is independently an optionally substituted 5 to 7 membered carbocyclic or heterocyclic ring;

R^1 where present is independently hydrogen, optionally hydroxylated, optionally alkoxylated alkyl optionally carrying a group CO-G where G is OR^2 or NR^2_2 and where Z is phosphorus optionally also oxo;

R^1 and R^1 which may be the same or different each

independently is hydrogen, optionally alkoxylated, optionally hydroxylated alkyl, aryl, alkaryl or aralkyl or R^3 may also represent or be substituted by a group CO-G; and NR^2_2 may also represent a nitrogen-attached optionally substituted 5 to 7 membered heterocyclic ring optionally containing a further nitrogen, oxygen or sulphur ring heteroatom; and where in place of two CR^2R^3groups, separated in either direction by at least one Z group, there may optionally be a bridging structure of formula



where u, g, h, i, j, k, l, m, n, q, r, s and t is each independently zero or a positive integer; p is a positive integer; h+l+j+n ≥ 1; and each D is independently boron, carbon, nitrogen or phosphorus or PO.

13. A compound according to any one of claims 1 to 11 wherein said macrocyclic chelants are selected from the residues of polyazacycloalkanepolycarboxylates, derivatized crown ethers, derivatized hexaazamacrocycles (HAMs), and derivatized cryptates, sepulchrates and sarcophagines.

14. A compound according to any one of claims 1 to 13 wherein said macrocyclic chelants are selected from the residues of 1,4,7,10- tetraazacyclododecanetetraacetic acid (DOTA), 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A), 1-oxa-4,7,10-triazacyclododecane-triacetic acid (DOXA), 1,4,7-triazacyclononanetriacetic acid

(NOTA), 1,4,8,11-tetraazacyclotetradecanetetraacetic acid (TETA), DOTA-N(2-aminoethyl)amide and DOTA-N(4-aminophenethyl)amide.

15. A compound according to any one of claims 1 to 14 comprising at least one said macrocyclic chelant-carrying backbone moiety conjugated to a site-directed molecule, or a chelate or salt thereof.

16. A compound according to claim 15 comprising a site-directed macromolecule capable of travelling to or binding specifically to targeted cells, tissues, organs or other locations in a mammalian body having conjugated thereto 1,2,3 or 4 said macrocyclic chelant moiety carrying backbone moieties, or a chelate or salt thereof.

17. A compound according to claim 15 wherein said site-directed molecule is selected from antibodies specific for a desired antigen, polymerized fibrin fragments, serum amyloid precursor proteins, low density lipoprotein precursors, serum albumin, surface proteins of intact red blood cells, hormones, liver-specific macromolecules, receptor binding proteins, fibrinogen, amino acids, oligopeptides, MRUs, SCAs, Fab fragments, polysaccharides, lectins, clotting factors and blood clotting proteins.

18. A compound according to claim 17 wherein said site-directed molecule is a monoclonal antibody specific for a desired antigen.

19. A compound according to any one of claims 15 to 18 wherein said site-directed molecule is bound to said backbone moiety by a heterobifunctional linking agent bonded via reactive linking groups selected from the group consisting of amide, maleamide, disulfide,

isocyanate, thiourea, isothiocyanate, and ester groups.

20. An image enhancing or therapeutic composition comprising a metal chelate of a polychelant according to any one of claims 1 to 19, or a salt thereof, together with at least one pharmaceutical carrier or excipient.

21. A method of generating an image of a human or non-human animal body, which method comprises administering to said body a polychelate according to any one of claims 1 to 19, or a salt thereof, and thereafter generating an image of at least a part of said body.

22. A method of radiotherapy of the human or animal body, said method comprising administering to said body a radioactive metal chelate of a polychelant according to any one of claims 1 to 19.

23. A detoxification composition comprising a polychelant according to any one of claims 1 to 19 or a weak chelate complex or salt thereof with physiologically tolerable counterions, together with a pharmaceutical carrier or excipient.

24. A method of metal detoxification comprising administering to a human or non-human animal a detoxifying amount of a polychelant according to claim 1 or a weak chelate complex or salt thereof with physiologically tolerable counterions.

25. A method of producing a polychelant according to claim 1 or a chelate thereof, said method comprising conjugating a plurality of macrocyclic chelants to an up to fifth generation dendrimeric polymer, optionally metallating the resultant polychelant, and optionally conjugating the polychelant or a chelate thereof to a site-directed molecule.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/02308

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K49/00; A61K47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X,Y	EP,A,0 430 863 (SCHERING AG.) 5 June 1991 see claims 1-2; example 22	1-25
P,X,Y	WO,A,9 012 050 (COCKBAIN J. R.) 18 October 1990 see claims 1,2,9,10,33	1-25
Y	EP,A,0 233 619 (NIHON MEDI-PHYSICS CO.) 26 August 1987 see claims; examples 1,5	1-25
X	WO,A,9 014 881 (AKZO N. V.) 13 December 1990 see page 5, line 17 - line 21 see page 7, line 31 - page 8, line 3; claims 19,26,30	1-25

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 FEBRUARY 1993	10. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BERTE M.J.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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X	WO,A,8 801 180 (THE DOW CHEMICAL CO.) 25 February 1988 see page 11, line 13 - page 12, line 7 see page 15, line 1 - line 14 see page 17, line 14 - line 27 see page 56; example DD	1-25
X	WO,A,8 801 179 (THE DOW CHEMICAL CO.) 25 February 1988 see claims 1-5	1-25
X	WO,A,8 801 178 (THE DOW CHEMICAL CO.) 25 February 1988 see claims	1-25
E	EP,A,0 512 661 (SCHERING AG.) 11 November 1992 see claims	1-25

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9202308
SA 66187

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 25/02/93
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		AU-A-	8139191	03-10-91
		EP-A-	0271180	15-06-88
		JP-T-	63502350	08-09-88
		JP-T-	63501876	28-07-88
		JP-T-	63501878	28-07-88
		WO-A-	8801178	25-02-88

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9202308 SA 66187

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		JP-T-	63501876	28-07-88
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